Intermittent Cyclic Stretch in Engineered Ligaments Drives Hierarchical Fiber Development and Crimp Formation

Leia D. Troop*[1], Jennifer L. Puetzer[1]
[1]Virginia Commonwealth University, Richmond, VA
troopld@vcu.edu

Disclosures: Leia D. Troop (N), Jennifer L. Puetzer (N)

INTRODUCTION: Hierarchical collagen fibers are the primary source of strength in tendons and ligaments. Injuries to these tissues disrupt the collagen organization resulting in loss of function.[1] These collagen fibers do not regenerate after injury or with repair.[1,2] Engineered replacements are promising, however, it remains a challenge to form the large hierarchically organized, crimped collagen fibers essential to long-term mechanical success. Previously, we developed a novel culture system that guides Anterior Cruciate Ligament (ACL) fibroblasts in high density collagen gels to develop native-sized hierarchically organized collagen fibers over 6 weeks of culture, however, further development is needed to be clinically relevant. Mechanical cues, including cyclic muscle activity, are critical for tissue development *in vivo* and have been shown to improve maturation of engineered tissues;[2,3,4] however the effect on hierarchical fiber formation is largely unknown. The objective of this study was to investigate whether intermittent cyclic stretch, mimicking rapid muscle activity, could drive further maturation in our system. Last year we reported that intermittent loading at 5 and 10% strain significantly improved collagen organization and tissue mechanics; however, 10% load drove early improvements in mechanics and composition, and 5% load was more beneficial later in culture (Fig 1C), suggesting a threshold in cellular response.[5] Here, we further evaluated hierarchical collagen organization and crimp formation in this system to see whether 5% load was resulting in similar significant improvements later in culture. We hypothesize that intermittent cyclic loading at 5% strain will improve overall collagen organization, composition, and tissue mechanics, resulting in significantly stronger, functional ligament replacements.

METHODS: To form constructs, rat tail type I collagen and neonatal bovine ACL fibroblasts were mixed and cast into 1.5 mm thick sheet gels at 20 mg/mL collagen and 5x10^6 cells/mL.[1] Rectangles (8 x 30 mm) were cut from gels, clamped into a modified CellScale tensile bioreactor (Fig. 1A), and cultured for up to 6 weeks. Static constructs were not loaded, while loaded constructs were stretched with an established loading regime[4,5] to mimic rapid muscle movement. Specifically, constructs were loaded with either 5% or 10% strain at 1 Hz for 1 hr, twice daily, every other day (Fig. 1B).[4,5] Zero-week samples were collected 24 hours after one loading cycle. Post culture, confocal reflectance was performed at all timepoints to track general collagen organization throughout culture. 6-8 images from each construct were analyzed via a custom Fast Fourier transform based MATLAB code[1,4] to determine degree of alignment (1 = unorganized, 4.5 = aligned) and fiber diameter. Six week constructs were further analyzed via SEM and Picrosirius red under polarized light to evaluate fibril and fascicle length-scale organization, respectively. SEM images were analyzed using the FIJI directionality plugin to determine degree of dispersion (n=6 images per construct) and fibril diameter (n=120 fibrils per construct). Neonatal bovine ACL was analyzed similarly for comparison. Mechanics were analyzed by tensile tests at 0.75% strain/sec to failure. All data are mean ± SD. Significance was determined via 1- and 2-way ANOVA with Tukey's post-hoc (p<0.05).

RESULTS: Looking first at fiber-level organization with confocal reflectance, as expected, static clamping guided cells to produce aligned collagen fibrils by 2 weeks and larger fibers by 4 and 6 weeks (Fig 2A, 2&4 wk data not shown). Cyclic loading appeared to further increase fiber maturation by inducing crimp formation by 2 weeks and more consistent, uniform crimp by 6 weeks (Fig. 2A). Image analysis of confocal images throughout culture further confirmed this with all constructs matching native alignment by 2 weeks, and cyclic loading further improving alignment at 2 and 4 weeks (Fig 3A). Further, all constructs had significant improvements in fiber diameter throughout culture, with both cyclic loaded groups having significantly larger fiber diameters compared to static controls at 4 and 6 weeks, matching native tissue diameters at 28-32 µm (Fig. 3A). However, there was no difference in fiber diameters between 5 and 10% load, despite differences in collagen concentration at 6 weeks (Fig 1C). We then further evaluated hierarchical organization in 6-week constructs. SEM analysis at the fibril level revealed that 5% and 10% load produced aligned fibril bundles by 6 weeks, similar to native tissue (Fig 2B). Image analysis of SEM further confirmed that fibrils in loaded constructs were less dispersed (i.e. more aligned) and had significantly larger diameters than static constructs, with some fibrils in loaded constructs reaching native size (~60 nm) (Fig. 3B). Again, there was no differences between 5 and 10% load. Picrosirius analysis at the fascicle level revealed 5 and 10% load produced larger fascicle bundles and more distinct crimp by 6 weeks (Fig. 2C). Mirroring the crimp formation, both 5 and 10% had significantly increased toe moduli compared to the static group at 6 weeks. Further, despite 5 and 10% producing similar sized fibrils and fibers, 5% had a significantly higher tensile modulus (Fig. 3C), mirroring collagen concentration at 6 weeks (Fig 1C).

DISCUSSION: Cyclic load has been shown to improve fibril level alignment, but its effect at the fiber and fascicle length scale is largely unknown. Here, we found that cyclic loading increased hierarchical collagen organization, collagen crimping, and tissue mechanics. Loaded constructs formed fibrils and fibers which matched native ACL alignment and diameter. Additionally, loaded constructs developed crimp by 6 weeks, as seen in imaging and reflected in mechanics, suggesting the development of crimp is leading to more functional tissues. Development of crimp is not well understood, but it is thought to be a reflection of the mechanobiological environment,[6] and in this study we see that both 5 and 10% load drive its development. Typically, 5% strain is used to stimulate maturation in engineered tendons and ligaments,[7] but in this case, 10% load outperformed 5%, potentially due to the ACL receiving larger strain *in vivo* (up to 10-12%).[8] Interestingly, while 10% load constructs had the largest, most aligned fibrils and fibers, it did not have as great of improvements in elastic modulus, which may be due to 5% load constructs accumulating significantly more collagen by 6 weeks. Collectively, this may suggest a threshold in cellular response for collagen production. Ongoing work is evaluating adaptive loads to drive further maturation.

SIGNIFICANCE: This study provides new insight into how cyclic loading affects cell-driven hierarchical fiber formation. A better understanding of how mechanical cues regulate fiber formation will help to better engineer replacements and develop better rehabilitation protocols to drive repair after injury.

REFERENCES:1.Puetzer+*Biomat* 2021; 2.Galloway+*J Bone Surg Am* 2013; 3.Connizzo+*Matrix Bio* 2013; 4. Puetzer+*Tis Eng* 2016; 5.Troop+*ORS Trans* 2023; 6.Lavagnino+JOR 2017; 7.Wang+JOR 2018; 8.Englander+J Biomech 2020

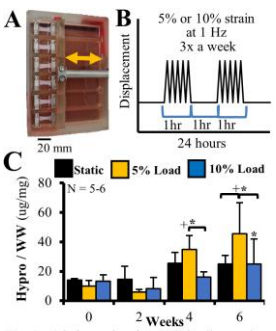

Fig. 1: A)Culture chamber & B)loading regime C)collagen (Hypro) / wet weight (WW). Significance to *0 wk & +bracket

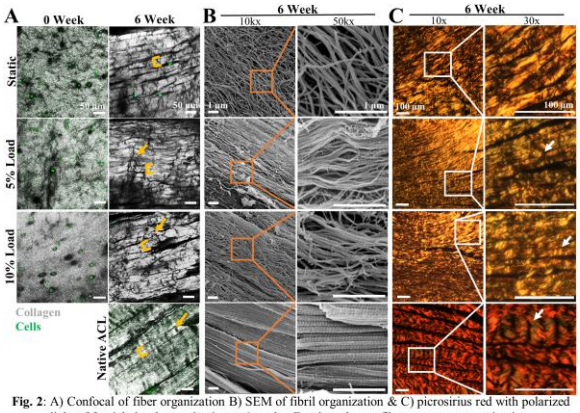

Fig. 2: A) Confocal of fiber organization B) SEM of fibril organization & C) picrosirius red with polarized light of fascicle level organization at 6 weeks. Brackets denote fibers, arrows note crimping.

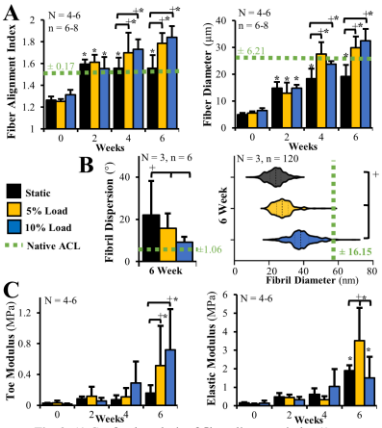

Fig. 3: A) Confocal analysis of fiber alignment index (1 = unorganized, 4.5 = aligned) & fiber diameter. B) SEM analysis of fibril dispersion & diameter. C) Construct tensile properties. Significance to *0 wk & +bracket